



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

TICKLE, et al.

Atty. Ref.: 620-282

Serial No. 10/690,991

Group: 1656

Filed: October 23, 2003

Examiner: NASHED, NASHAAT T

For: CRYSTAL STRUCTURE OF CYTOCHROME P450

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

RULE 132 DECLARATION

I, Pamela Ann Williams, declare as follows:

1. I am a Senior Research Associate for Astex Therapeutics Limited (previously called Astex Technology Limited), the assignee of the above-identified application. Prior to joining Astex Technology Limited, I worked at the University of Oxford, UK, on enzyme structure determination. Prior to this, I was a postdoctoral research assistant at The Scripps Research Institute and obtained a DPhil in Molecular Biophysics in X-ray protein crystallography from the University of Oxford in 1996. A copy of my curriculum vitae is attached hereto as an Exhibit.
2. I am a co-inventor of at least one claim of the above-identified application. I have reviewed the above-identified application (which I refer to below as the "present application"), including the attached pending claims, the attached claims and accompanying response which I understand are being submitted to the Patent Office with the present Declaration.
3. I have been advised by Astex's patent counsel that the U.S. Patent Office Examiner responsible for the above-identified application has asserted that the above-identified

application allegedly fails to teach one of ordinary skill in the art how to make and use the claimed invention and that the Examiner has asserted that the above-identified application allegedly fails to describe the claimed invention in a manner that would lead one of ordinary skill in the art to conclude that the applicants were in possession of the claimed invention at the time the application was filed.

4. I have been requested by patent counsel for Astex to provide the following technical comments relating to the state of the art of X-ray crystallography in general and specifically with regard to the presently claimed invention.

5. In addition to my work on the crystallization and structure determination of the P450 3A4 protein, I am also an inventor of the crystallization and structure determination of another cytochrome, P450 2C9. These proteins were the first two human cytochrome P450 structures to be determined, something which represented a significant advance in the scientific community. The structure of P450 3A4 reported in the present application was published in the leading journal Science (Williams et al; Vol. 305 30th July 2004 p 683-686). This publication includes data (i.e. the crystal of SEQ ID NO:2) of the present application. It is generally recognized in the art that to have a paper published in Science is a reflection of the very considerable importance of the present findings.

6. In view of my experience in the field of protein crystallization, particularly with human cytochrome P450 proteins, I consider myself to be an expert in the field.

7. P450 nomenclature gives each P450 a family (e.g. 3), a sub-family (e.g. 3A) and an individual gene (e.g. 3A4) based on sequence identity. Thus only identical genes and their alleles can be given the same name. For example 2C9 and 3A4 have $\leq 40\%$ sequence identity and therefore belong to different families '2' and '3'. 2C9 (human) and 2D6 (human) belong to the same family '2' so they have $> 40\%$ sequence identity. 3A4 (human) and 3A2 (rat) have $\geq 55\%$ sequence identity (71.8%) so belong to the same mammalian subfamily '3A' but have different names as they are genes from different species (i.e. homologous). Thus, while other 3A proteins are present in other mammals (e.g. 3A1 in rats and 3A6 in rabbits), 3A4 is a human

protein. Accordingly, reference in the claims to 3A4 protein would be understood by a person of skill in the art to be a reference to a **human** protein, and not to a protein from another mammalian species.

8. I understand from patent counsel for Astex that the Examiner has suggested that the P450 3A4 of the above-identified application is a glycosylated protein. One of ordinary skill in the art will appreciate however that P450 3A4 is a human protein which is not known to be glycosylated. To this extent, the production of P450 3A4 in a recombinant bacterial system will not produce a protein which is different from that produced in a native human cell.

9. Prior to the present invention, I was a co-inventor of crystals of various sequences of the human protein P450 2C9. International Patent Application WO 03/035693 describes some of the work referred to herein below, though this is set out herein with additional data.

10. I and my co-inventors were able to make crystals of human P450 2C9 after making a change to its N-terminal, adding a 4-histidine tag to the C-terminal, and introducing other modifications. I and my co-inventors made a number of protein sequence variants between these N- and C-terminal features. The variants for which crystal data are provided for are as follows:

Clone 1015; comprising alterations I215V C216Y S220P P221A I222L I223L;

Clone 1155; as 1015 plus K206E

Clone 1475; as 1155 plus N231H

Clone 1491; as 1155 plus L208A

Clone 1982; as 1155 plus A103Y

Clone 1983; as 1155 plus A103W

Crystals of these variants have all been produced (in duplicate for 1015 and 1155) and have space groups and unit cell sizes, to 2 decimal places, as follows:

2C9 Clone	Unit Cell	a	b	c
1015	P321	161.35	161.35	110.75
1015	P321	163.95	163.95	111.06
1155	P321	165.46	165.46	111.70
1155	P321	164.87	164.87	111.11
1475	P321	165.39	165.39	110.91
1491	P321	164.80	164.80	111.23
1982	P321	165.31	165.31	111.45
1983	P321	164.57	164.57	111.25

The cell sizes are all within 5% of each other. As a practicing crystallographer, a variation within this limit means to all intents and purposes the crystals have the same unit cell size and when I refer herein to crystals being of the same size, it is in this context of a practitioner in the art, rather than in the strictly mathematical sense. Thus 1 or 2 amino acid substitutions on the Clone 1015 core sequence did not change the cell dimensions or space group.

11. As an expert in the art, I consider it reasonable to believe that in general, at least in the art of P450 proteins, sequence variations to the primary amino acid sequence of a P450 protein of the type illustrated above are unlikely to affect the unit cell size or space group of the resulting protein crystal. My opinion is supported by the published data cited in the accompanying response relating to P450 BM3, P450cam and P450nor crystals, which show retention of unit cell size and space group for variations of the wild-type sequence. In the present application we teach that alleles of 3A4, which are often only different by one or two amino acids changes to the core sequence of P450 3A4, can be made (page 13, lines 23-24). This teaching is not mere speculation but, in my opinion as an expert in the art of P450 crystallization, variations of up to 2 residues in the fashion illustrated above, which those of ordinary skill in the art would reasonably contemplate in the light of the knowledge of crystals of other P450s.

12. One of ordinary skill in the art will appreciate that there are a number of naturally-occurring variations of the P450 3A4 amino acid sequences. In humans, these variations can amount to approximately 3% of the amino acid structure, as described in the attached Pharmacogenetics, Nelson et al, 1-42, 1996 reference. In view of the fact that crystals of

P450BM3, P450cam and P450nor proteins published in the literature, together with the published and unpublished data on P450 2C9 cited above, all form consistently sized unit cells within this degree of variation, my belief is that crystals of P450 3A4 incorporating a similar degree of sequence variation, having an orthorhombic space group I222, and unit cell dimensions $a=78 \text{ \AA}$, $b=100 \text{ \AA}$, $c=132 \text{ \AA}$, $\alpha=\beta=\gamma=90^\circ$, with a unit cell variability of 5% in all dimensions will generally be obtainable by following the teaching of the present application.

13. All P450 proteins have a tertiary core structure brought about by the folding of secondary structural features such as alpha-helices and beta-sheets. The N- and C-terminal regions outside the core do not interact strongly, if at all, with the core domains of our 3A4 structure and do not appear to play a significant role in the resulting three-dimensional structure of the protein. One of ordinary skill in the art will appreciate that the P450 3A4 amino acid sequences, like many proteins, contain a core structure which, for the purposes of crystallization and crystal structure elucidation, is generally the most relevant aspect of the crystallized product. For example, it is noted in the accompanying response that a P450 BM3 heme-domain crystal with the same unit cell size and space group was achieved using a P450 BM3 sequence whose C-terminal region was 16 amino acids shorter. I do not consider this unusual, as this region is outside the core part of P450 in terms of major P450 secondary structural domains and contains amino acids which were not resolved by X-ray crystallographic analysis. The fact that these residues were not resolved in the electron density maps indicates disorder in this region.

14. In the present application we have identified that residues 17-476 of SEQ ID NO:2 provide a structural core of the P450 3A4 protein. This is taught in the present application at page 25 lines 6-7, which state that the last resolvable residue is Gly498. This corresponds to 476 of SEQ ID NO:2, as "Gly 498" refers to wild-type (SwissProt M18907) numbering. We identified this as the end of the core since at the C-terminal end there are no resolvable residues after 476 of SEQ ID NO:2. At page 10 line 24 we identify the start of the core sequence as residue 17 of SEQ ID NO:2. This is because although some of the residues prior to 17 of SEQ ID NO:2 are resolved, these appear as a short helix - denoted A" - which has not previously been observed in mammalian P450 structures (taught at page 25 lines 17-18). This helix does not appear to have many significant interactions with any other secondary structural

features and in my opinion would not be expected to be of significance in the determination of how the rest of the protein folds.

15. In view of the identification of the core sequence of 3A4 identified in the application, it is my belief that others of ordinary skill in the art wishing to make 3A4 protein crystals would be guided by this identification. In deciding what protein sequence to express, they would be guided to select a core region of 17-476 and place this within short N- terminal (smaller than 20 amino acids in size) and C-terminal (no larger than 10 amino acids in size) regions as a matter of experimental convenience. A more conservative approach would be to utilize all the residues resolved in our structure, i.e. from 3 to 476, though my belief is that this would not be essential. Another approach would be to use from residue 3 to the last native 3A4 residue, 481, though again my belief as an expert in the art is that this would not be essential to obtain a crystal within unit cell dimensions of the present claims.

16. In summary, where a core region of a protein is resolved and contains amino acids which form a stable, resolvable, structure with identifiable secondary structural features, it is unlikely that those features would be affected by minor variations in N- or C-terminal regions which a person of skill in the art would be expected to modify as a matter of experimental convenience. These N- and C-terminal tails are unlikely to influence the structures of the more rigidly folded domains of the 3A4 protein itself.

17. The crystallization of P450 3A4 described in the present application and in the publication in Science (see §5 above) has been recognized by other practitioners in the art as being the structure of P450 3A4, regardless of the variations to the N- and C-terminal regions of the particular construct of SEQ ID NO:2. This is apparent from the description of the Science publication (i.e. of the crystal of SEQ ID NO:2 and its structure) by others of skill in the art. For example:

Nature Review Drug Discovery (2004, Vol. 3 No 9 p736-737) article about the Science paper stated "the crystal structure of arguably the most important enzyme in drug metabolism – cytochrome P450 3A4 (CYP3A4) – has been published"

Current Opinion in Structural Biology (2004, Vol. 14, Issue 6, p706-715) states "For example, in the recent crystal structure of cytochrome P450 3A4...." (Williams *et al* Science 2004 then cited).

In Drug Metabolism Reviews (2005, Vol. 37, p489-510) Mizutani et al stated "Recently, crystal structures of human CYP2C9 and CYP3A4 of major phase I drug-metabolising enzymes were clarified" (Williams *et al* Science 2004 then cited).

Journal of Molecular Biology (2005, Vol. 352, 165-177) states ".....recently in the structure of the human drug metabolism enzyme cytochrome P450-3A4. (Williams *et al* Science 2004 then cited)"

Journal Of The American Chemical Society, (2005, Vol. 127, No. 39, pp. 13634-13642) states 'Recently, Williams *et al.* reported X-ray crystal structures of CYP3A4....' (Williams *et al* Science 2004 then cited)

All of the above citations refer to the P450 3A4 structure in general terms, and do not suggest that the core structure of this protein would be any different in the wild-type native enzyme, nor in variants such as those allelic variants known in the art. In my opinion, if those of skill in the art had felt that the crystal of SEQ ID NO:2 and its resulting structure was peculiar only to this specific sequence the publication in Science would not have been cited as widely or as rapidly as it has been. The value of the publication is that it provides others of ordinary skill in the art the means, in the light the general state of knowledge in the art of P450 crystallization, to crystallize P450 3A4 proteins such as allelic variants and those with N- and C-terminal regions made suited to the particular preference of any one particular laboratory. The present application contains more extensive teaching than the Science paper and is thus equally applicable across a broad range of P450 3A4 sequences.

18. In the present application we set out at page 11 lines 15-22 general guidance to those of ordinary skill in the art relating to conditions we have discovered are suitable for the crystallization of P450 3A4. As a person of skill in the art, it would be my understanding that this teaching was intended to apply to the class of P450 3A4 proteins set out on the preceding

pages, namely proteins which retained the 3A4 core sequence of SEQ ID NO:2 as well as variants of SEQ ID NO:2 having up to two amino acid substitutions, such as allelic variants. A person of ordinary skill in the art should be able to use this teaching, together with the other information in the present application (e.g. that in section (iii) starting at page 13 line 36) to obtain, *inter alia*, crystals of P450 3A4 protein having an orthorhombic space group I222, and unit cell dimensions $a=78 \text{ \AA}$, $b=100 \text{ \AA}$, $c=132 \text{ \AA}$, $\alpha=\beta=\gamma=90^\circ$, with a unit cell variability of 5% in all dimensions, wherein said 3A4 protein comprises the core of residues 17 to 476 of SEQ ID NO:2, as well as a crystal of P450 3A4 having an orthorhombic space group I222, and unit cell dimensions $a=78 \text{ \AA}$, $b=100 \text{ \AA}$, $c=132 \text{ \AA}$, $\alpha=\beta=\gamma=90^\circ$, with a unit cell variability of 5% in all dimensions, wherein said P450 3A4 is of SEQ ID NO:2 or comprises from 1 to 2 amino acid substitutions or deletions thereof.

19. I understand from patent counsel for Astex that the Examiner has suggested that one of ordinary skill in the art would require distinct crystallization conditions for each different "primary structure of the P450 3A4".

For the reasons set out in the paragraphs 10-18, one of ordinary skill in the art will appreciate that, given the above-identified application, in the light of the teaching of the present application, no more than routine experimentation would be required to crystallize P450 3A4 proteins containing naturally-occurring variations of, and sequences with different N- and C-termini attached to, the core structure of P450 3A4.

20. I further understand from patent counsel for Astex that the Examiner has stated the following:

"Since routine experimentation in the art does not include screening large number of crystallization conditions for the wild-type P450 3A4 or modified form thereof which can be crystallized where the expectation of obtaining the desired crystal is unpredictable, the Examiner finds that one skilled in the art would require additional guidance, such as information regarding the amino acid sequences of P450 3A4 to be crystallized and the exact crystallization

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conditions that produce a crystal suitable for structure determination by X-ray crystallography and having the crystal parameters cited in claims 98 and 101."

As an expert in the art of P450 crystallization, I consider this statement to be a misrepresentation of the state of the art. Having identified several suitable conditions for the crystallization of the P450 3A4 protein whose sequence is set out in the present application, it is my belief, based on my own experience as an expert in the field and the literature relating to other P450 proteins, that those of ordinary skill in the art would expect to be able to use these conditions for the crystallization of many different variants of P450 3A4, including naturally occurring alleles as well as proteins whose C- and N-terminals outside the core region had been modified, for example to an individual's preferred choice of purification tag or N-terminal sequence adapted for a preferred choice of expression host.

I declare further that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By 

Pamela Ann Williams

Date: December 19th, 2005

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Pending claims

98. (Previously Presented) A crystal of P450 3A4 having an orthorhomobic space group I222, and unit cell dimensions 78 Å, 100 Å, 132 Å, 90°, 90°, 90°, with a unit cell variability of 5% in all dimensions.

99. (Previously Presented) The crystal of claim 98 wherein said crystal comprises the sequence of SEQ ID NO:2

100. (Previously Presented) A crystal of P450 3A4 protein having the structure defined by the co-ordinates of Table 5 + a root mean square deviation from the Ca atoms of not more than 1.5 Å.

101. (Previously Presented) A crystal of P450 3A4 having a space group I222 and unit cell size a=77 Å, B=99 Å, C=129 Å, (+/-5% for a, b and c), $\beta=90^\circ$.